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Heterotrophic $^{15}N_2$ Fixation and Distribution of Newly Fixed Nitrogen in a Rice-Flooded Soil System[1]

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ABSTRACT

Rice (*Oryza sativa* L.) plants growing in pots of flooded soil were exposed to a $^{15}N_2$-enriched atmosphere for 3 to 13 days in a gas-tight chamber. The floodwater and soil surface were shaded with a black cloth to reduce the activity of phototrophic N_2-fixing micro-organisms. The highest ^{15}N enrichments were consistently observed in the roots, although the total quantity of ^{15}N incorporated into the soil was much greater. The rate of ^{15}N incorporation into roots was much higher at the heading than at the tillering stage of growth. Definite enrichments were also found in the basal node and in the lower outer leaf sheath fractions after 3 days of exposure at the heading stage. Thirteen days was the shortest time period in which definite ^{15}N enrichment was observed in the leaves and panicle. When plants were exposed to $^{15}N_2$ for 13 days just before heading and then allowed to mature in a normal atmosphere, 11.3% of the total ^{15}N in the system was found in the panicles, 2.3% in the roots, and 80.7% in the subsurface soil. These results provide direct evidence of heterotrophic N_2 fixation associated with rice roots and the flooded soil and demonstrate that part of the newly fixed N is available to the plant.

Nitrogen fixation by heterotrophic bacteria associated with rice (*Oryza sativa* L.) growing in flooded soil has been extensively studied, and this work has been summarized in a series of reviews (7, 12, 16). Most of these studies in the field and in the laboratory have used the acetylene reduction assay, and activity has been found in the lower portion of the stem, the roots, the rhizosphere soil, and in the bulk of the anaerobic soil (8, 15, 17, 19). There have also been several studies which demonstrated incorporation of $^{15}N_2$ into flooded rice soils without plants (5, 11, 14, 19). Recently, Ito *et al.* (9) demonstrated incorporation of $^{15}N_2$ into the roots, basal node, and decaying outer leaf sheaths of rice plants which had been transferred from the field and exposed to $^{15}N_2$ in N-free nutrient solution. These studies do not allow direct comparison of the activity in the soil *versus* that in the roots, and there is also no information on the fate of the fixed N. There have been few experiments done in which undisturbed grass soil systems were exposed to $^{15}N_2$ and none with rice. De-Polli *et al.* (6) showed that the roots and rhizomes of *Digitaria decumbens* and *Paspalum notatum* became enriched when soil cores containing these plants were exposed to $^{15}N_2$ and presented some evidence for transport of the newly fixed nitrogen to the leaves of *P. notatum*. In the present study, rice plants growing in pots of flooded soil were exposed to $^{15}N_2$, and the incorporation of ^{15}N into the plant-soil system and its subsequent redistribution with time was investigated.

MATERIALS AND METHODS

Plant Culture. Seeds of rice (*Oryza sativa* L.) cultivar IR 2037-117-2 were germinated on plastic screens floating on a 100:1 dilution of the nutrient solution described by Yoshida *et al.* (18). Ten-day-old seedlings were transplanted into glazed pots (23 cm i.d.) containing approximately 5.5 kg dry weight Maligaya clay soil (pH 6.6). This is a soil used in rice production in Central Luzon, the Philippines, and was obtained from the Maligaya Rice Research Training Center in Nueva Ecija. Three seedlings were transplanted into each pot for experiments 1 and 2, and two per pot for experiments 3 and 4. The floodwater and surface soil were shaded by a black felt cloth stretched over the top of each pot to limit development of phototrophic microorganisms. The plant leaves extended through a slit in the cloth. Plants were grown in a controlled environment chamber (Environmental Growth Chambers, Chagrin Falls, OH) with a 12-h photoperiod. A PPFD[3] of 550 μE m^{-2} s^{-1} at pot level (400–700 nm, measured with a LI 190S quantum sensor, Li-Cor, Lincoln, NB) was provided by a 1:1 mixture of General Electric 400 w Lucalox^R lamps and 400 w Multivapor^R lamps. The day to night temperature regime was 28 to 21 C, and RH was maintained between 60 and 80%.

Exposure Apparatus. A diagram of the $^{15}N_2$ exposure apparatus is shown in Figure 1. The chamber (A) enclosing the pot consisted of a 29.3 cm (i.d.) × 24.5 cm piece of aluminum tubing fitted with end flanges of 1.3-cm-thick aluminum plate. A groove (0.7 cm deep) to fit the end of the tubing was machined into each flange and Buna-N O rings were used to make a gas-tight seal. The plant shoot was enclosed in a borosilicate glass dome (B) made by closing off one end of a 15.2 cm (i.d.) × 60 cm length of QVF pipe (Mooney Bros., Little Falls, NJ). A H_2 burner (C) was added to the apparatus for the fourth experiment to control the accumulation of O_2. A stainless steel condenser (D), cooled to 1 C, was used to remove moisture from the circulating gas, and the condensed water was returned to the floodwater in the pot through tube E. Some condensation still occurred inside the plant chamber where leaves touched the glass surface. An MB-118 (Metal Bellows Co., Sharon, MA) pump (F) was used to circulate the atmosphere at 21 liters min^{-1}, as measured by a rotameter (G). Two manually adjusted valves (H) were used to divert approximately 600 ml min^{-11} through the IRGA (I). The IRGA output was used to remotely control the valves at J and L. During the light period, valve L was automatically opened to admit pure CO_2 through a capillary, and during the dark period, the two valves at J were automatically switched to pass the gas returning from the IRGA through a soda lime column (K) to maintain a CO_2 concentration

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[3] Abbreviations: PPFD, photosynthetic photon flux density; IRGA, infrared gas analyzer; DAT: days after transplanting.

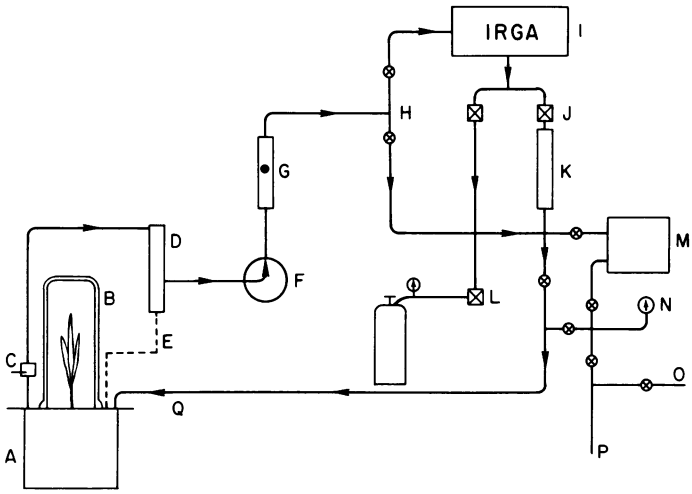

FIG. 1. Diagram of the apparatus used to expose the plant-soil system to a $^{15}N_2$-enriched atmosphere. A, aluminum chamber enclosing pot; B, QVF glass pipe-dome enclosing shoot; C, H_2 burner; D, stainless steel condensor; E, condensed water return; F, MB-118 pump; G, rotameter; H, manual control valves; I, IRGA; J, automatically controlled valves; K, soda lime column; L, automatically controlled valve and tank of pure CO_2; M, 5 liter reservoir; N, pressure-vacuum gauge; O, to vacuum pump; P, sampling port; Q, gas return to chamber.

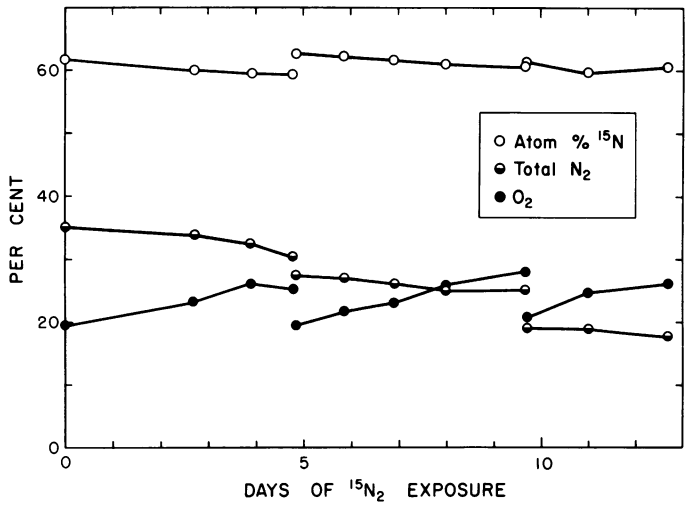

FIG. 2. Gas composition of the atmosphere during the exposure period, Experiment 3. By day 5, pressure inside the chamber had risen to 1.2 atm due to O_2 evolution. At this point, the chamber was vented to atmospheric pressure, and then 4 liters of Ar and 1 liter of $^{15}N_2$ were introduced using the reservoir (M) to lower the O_2 concentration. On day 10, pressure had reached 1.2 atm, the chamber was again vented, and 5 liters of Ar were added. At the end of the exposure, pressure had increased to 1.1 atm.

of 325 $\mu l\ l^{-1}$. A Beckman 15A IRGA was used in experiments 1 and 2, but it was replaced with a Beckman 815 for experiments 3 and 4.

A 5-liter reservoir (M) which could be isolated from the rest of the apparatus was used to introduce Ar, O_2, and $^{15}N_2$. The reservoir was isolated and evacuated (monitored by vacuum-pressure gauge [N]) through 0, then filled with Ar through P, and finally the 21 l min^{-1} flow was diverted through M and back to the plant chamber through Q. This sequence was repeated six times with Ar, once with O_2, and once with $^{15}N_2$. This procedure was used so that Ar and $^{15}N_2$ could be introduced with a minimum of disturbance to the plant. Figure 2 shows an example of the gas mixture achieved by this method. Enriched $^{15}N_2$ (99%+) gas was purchased from Monsanto Research Corp. (Miamisburg, OH) and treated to remove oxides of N and NH_3 with the reagents described by Burris (3). Gas samples were collected with 15-ml glass vials equipped with high vacuum stopcocks. Vials were connected at P, evacuated through 0, and then allowed to equilibrate with the chamber atmosphere. Samples were analyzed for Ar, O_2, N_2, $^{15}N_2$, and oxides of N with a VG Micromass 622 MS (VG Isotopes Limited, Winsford, Cheshire, England).

Exposure Conditions and Plant Sampling. The apparatus was placed inside a controlled environment chamber identical with the one in which the plants had been grown. The PPFD inside the glass dome varied from 670 to 489 $\mu E\ m^{-2}\ s^{-1}$ from the top to the bottom. The same 12-h photoperiod that the plants were grown in, from 6:30 a.m. to 6:30 p.m., was used during the exposures. By adjusting the air temperature and circulation around the outside of the sealed plant chamber, air temperature inside the chamber was held at a day to night regime of 28 to 21 C (measured with a thermistor probe). Soil temperature varied slowly from a maximum of 27 C at the end of the light period to a minimum of 22 C near the end of the dark period.

Each of the four experiments performed in this study differed in the length of exposure to $^{15}N_2$, gas composition, and in the procedure for fractionating the plant into samples for analysis. The details of each exposure are provided in the tables. No differentiation was made among the original seedlings in sampling. In all experiments the shoot was divided into upper and lower shoot samples at the level of the black cloth, because the part of the shoot below the black cloth was partly submerged in the flood water and exposed to light penetrating through the slit in the black cloth. Thus, ^{15}N enrichments may have represented both phototrophic and heterotrophic fixation.

Because of the pressure increases inside the chamber during Experiment 3, influx of CO_2 from the capillary was prevented for periods of 1 to 2 h on two occasions during the exposures. All the flag leaves died during the exposure, but whether this was due to the CO_2 control problem or another problem has not been determined. The dry weight of the panicles at maturity was reduced to 14.6 g in comparison to 25.4 ± 1.9 g for five unexposed pots of rice grown under otherwise similar conditions. At the end of the

Table I. *Experiment 1. ^{15}N Enrichment of a Rice-Soil System Exposed to $^{15}N_2$ for 6.8 Days (5:35 p.m. 19 DAT-1:15 p.m. 26 DAT).*

Plants were in the tillering stage. During the exposure, O_2 concentration increased from 15–25%, total N_2 remained constant at 33% but atom % ^{15}N declined from 79–63%, and Ar decreased from 53–43%. Pressure inside the chamber rose to 1.1 atm. The whole plant was harvested for analysis immediately after the exposure.

Sample	Dry wt	Total N	^{15}N	^{15}N
	g	*mg*	*atom % excess*[a]	*mg*
Above black cloth				
1st leaf blade	1.01	40.0	−0.002 ± 0.001 (8)	
2nd leaf blade	0.95	31.1	0.001 ± 0.005 (8)	
3rd leaf blade	0.27	7.9	−0.002 ± 0.001 (3)	
Outer sheaths	0.54	7.9	0.000 ± 0.003 (8)	
Inner sheaths	0.73	20.0	0.000 ± 0.003 (8)	
Dead blades	0.11	5.2	0.001 ± — (1)	
Below black cloth				
Outer sheaths	0.42	5.3	0.000 ± 0.002 (3)	
Basal node + inner sheaths	0.62	19.7	0.000 ± 0.001 (3)	
Roots	1.1	11.9	0.023 ± 0.003 (2)	0.003
Surface soil	169	118.7	0.017 ± 0.003 (4)	0.02
Subsurface soil	5970	3742.6	0.005 ± 0.002 (4)	0.19

[a] Values are relative to unexposed plant and soil samples. Standard deviations calculated with the number of samples indicated in parentheses.

Table II. *Experiment 2. ^{15}N Enrichment of a Rice-Soil System Exposed to $^{15}N_2$ for 3 Days (2:11 p.m. 46 DAT-2:15 p.m. 49 DAT).*

Plants were in the heading state. During the exposure, O_2 concentration increased from 17–23%, total N_2 decreased from 32–31%, atom % ^{15}N decreased from 65–60%, and Ar decreased from 51–46%. Pressure inside the chamber rose to 1.03 atm. The whole plant was harvested immediately after the exposure.

Sample	Dry wt	Total N	^{15}N	^{15}N
	g	*mg*	*atom % excess*[a]	*mg*
Above black cloth				
Panicles	2.85	29.3	0.000 ± 0.001 (6)	
Flag leaves	2.46	28.8	0.000 ± 0.001 (6)	
2nd leaves	3.14	23.9	0.001 ± 0.001 (5)	
3rd and 4th leaves	6.60	34.9	0.000 ± 0.002 (6)	
Stem	2.59	11.9	0.001 ± 0.002 (6)	
Young tillers	3.06	32.7	0.001 ± 0.000 (2)	
Below black cloth				
Outer sheaths	0.84	1.9	0.066 ± 0.002 (2)	0.001
Inner sheaths + stems	5.73	14.7	0.005 ± 0.001 (2)	
Basal nodes	1.10	4.7	0.025 ± 0.002 (2)	0.001
Surface roots	0.34	2.0	0.054 ± — (1)	0.001
Subsurface roots	4.70	25.1	0.216 ± 0.001 (3)	0.054
Surface soil	169	111.0	0.009 ± 0.002 (4)	0.01
Subsurface soil	5580	3533.0	0.008 ± 0.002 (4)	0.28

[a] Values are relative to unexposed plant and soil samples. Standard deviations calculated with the number of samples indicated in parentheses.

Table III. *^{15}N Enrichment of Single Tillers Harvested Immediately after Exposure in Experiments 3 and 4.*

See Tables IV and V for exposure details.

Sample	Dry Wt	Total N	^{15}N
	mg		*atom % excess*[a]
Experiment 3			
Above black cloth			
Panicle	19	0.79	0.065
Dead flag leaf	219	3.18	0.045
2nd leaf	462	7.11	0.031
3rd leaf	203	2.11	0.012
Dead lower leaf	176	1.13	0.007
Below black cloth			
Outer sheath	146	0.47	0.042
Inner sheath	80	0.77	0.091
Experiment 4			
Above black cloth			
Panicle	1089	9.47	0.003
Flag leaf	350	2.78	0.000
2nd leaf	374	1.61	−0.002
Dead leaf	167	0.51	0.000
Below black cloth			
Outer sheath	202	0.30	0.012
Inner sheath	387	0.07	0.002

[a] Values are relative to unexposed plant samples.

exposure in both experiments 3 and 4, one tiller was cut off at the soil surface and divided into samples for analysis. The pot was then returned to the first controlled environment chamber and allowed to mature in a normal atmosphere. At 79 DAT several of the panicles were mature and these were harvested to prevent loss of grain. The rest of the plant was harvested at 87 DAT.

Soil and Root Sampling. Soil and root samples were collected at the end of the exposure in experiments 1 and 2 and at 87 DAT in experiments 3 and 4. After most of the flood water had been removed, the surface 0.5 cm of soil was collected and passed through a 1-mm sieve to remove roots, and the resulting soil slurry was held at −20 C until analysis. In experiments 2 to 4, enough roots were recovered from the surface soil during sieving to allow a separate total N and ^{15}N analysis; this fraction was called surface roots. The bulk of the root system was recovered while still attached to the shoot. Some additional roots were recovered from the soil by hand. The combined roots were then washed three times with deionized H_2O. The subsurface soil was thoroughly mixed and a sample was sieved and stored for analysis. Samples were also collected for determination of % dry weight and total weight of soil was calculated.

Total N and ^{15}N Analysis. Plant samples were dried for 48 h in a forced draft oven at 60 C. Total N was determined by Kjeldahl digestion as described by Bremner (2). No nitrate was found in preliminary digestions, and steps to include NO_3^- in the analyses were omitted. Soil samples were diluted with deionized H_2O and stirred with a magnetic stirrer until they could be pipetted smoothly with a 10-ml serological pipet. Thirty-ml samples containing approximately 10 g dry weight of soil were pipetted into 800-ml Kjeldahl flasks and digested as described by Bremner (2), omitting steps for nitrate. Four subsamples were run from each soil sample. After titration, the samples were acidified to pH 3 with 0.1 N H_2SO_4, dried, transferred to 16 × 50 mm vials, and dried again. Samples were oxidized to N_2 with LiOBr with a system similar to that described by Porter and O'Deen (13) and analyzed for ^{15}N concentration with a Micromass 622 MS. Atom % ^{15}N values for unexposed plant and soil samples were subtracted from the experimental values to calculate atom % ^{15}N excess.

RESULTS AND DISCUSSION

Direct evidence for heterotrophic nitrogen fixation associated with the roots of rice was obtained in all four experiments (Tables I, II, IV, and V). Even the comparatively low root ^{15}N enrichment found in experiment 1 exceeded the 0.015 atom % ^{15}N excess level set by Burris and Wilson (4) as a conservative standard for establishing nitrogen fixation. The black cloth treatment was effective in controlling the growth of blue-green algae; the floodwater was always clear and there was no visual evidence of algal development on the surface of the soil. Further evidence that the root enrichments were due to heterotrophic fixation and not due to phototrophic fixation and subsequent transfer of fixed N to the roots was found by comparing the enrichments of roots from the surface soil and the roots from the subsurface soil. In experiments 2 and 3, the subsurface roots were more enriched than the surface roots, and in experiment 4, they were equally enriched. This pattern of enrichments could not be explained by phototrophic fixation.

The apparent rate of nitrogen fixation in rice roots was much higher at the heading stage than at the tillering stage. A heading stage plant exposed to $^{15}N_2$ for 3 days had a much higher root ^{15}N enrichment than a tillering stage plant exposed for 6.8 days (Tables I and II). Considering the differences in exposure duration, total N per root system and atmosphere ^{15}N enrichment, these results represent a 50-fold increase in nitrogen fixation per plant per day. Yoshida and Broadbent (20) have shown that diffusion of $^{15}N_2$ from the gas phase, through the shoot, and to the roots of rice is much faster in heading stage plants than in tillering stage plants. Thus, part of the increase in ^{15}N incorporation rate may be due to more rapid diffusion of $^{15}N_2$ to the roots in the heading stage plant. These results are based on only two experiments, and plant variability could represent a significant part of the difference in activity. Similar patterns of acetylene reduction activity have been reported using *in situ* assays (10, 17) and excised root assays (19).

There was no evidence of phyllosphere fixation in leaves above the black cloth. No enrichment was found in the leaves of the plants after the exposures of experiments 1, 2, and 4, and only in the 13-day exposure of experiment 3 were the leaves enriched with

Table IV. *Experiment 3. ^{15}N Enrichment in a Rice-Soil System Exposed to $^{15}N_2$ for 12.8 Days (3:30 p.m. 31 DAT-10:00 a.m. 44 DAT, Tillering Stage), and Harvested at Maturity (87 DAT).*

The composition of the atmosphere during this exposure is shown in Figure 2.

Sample	Dry Wt	Total N	^{15}N	^{15}N	Total ^{15}N
	g	*mg*	*atom % excess*[a]	*mg*	*%*
Above black cloth					
Early panicles[b]	4.60	54.7	0.284 ± 0.001 (3)	0.156	3.5
Panicles	10.07	99.5	0.344 ± 0.001 (4)	0.343	7.8
Shoot	16.51	65.1	0.141 ± 0.007 (7)	0.092	2.1
Below black cloth					
Lower shoot	2.94	5.8	0.273 ± 0.005 (3)	0.016	0.4
Basal node	1.08	4.2	0.502 ± 0.003 (3)	0.021	0.5
Surface roots	0.11	0.8	0.441 ± 0.058 (2)	0.004	0.1
Subsurface roots	2.68	10.9	0.898 ± 0.012 (3)	0.097	2.2
Surface soil	169	108.0	0.116 ± 0.002 (4)	0.13	2.8
Subsurface soil	5379	3244.0	0.110 ± 0.003 (4)	3.57	80.7

[a] Values are relative to unexposed plant and soil samples. Standard deviations calculated with the number of samples indicated in parentheses.

[b] The early maturing panicles were harvested at 79 DAT.

^{15}N during the exposure. Watanabe *et al.* (17) have reported high acetylene reduction activity in the lower part of the stem. Some enrichment of the outer leaf sheath below the black cloth and the basal node of the stem was observed in experiment 2, but the level of enrichment was lower than in the roots. In contrast, Ito *et al.* (9) found higher nitrogen fixation activity in the outer leaf sheaths than in the roots. These discrepancies might be due to differences in the rice cultivars used in the two studies or to differences in experimental technique. Ito *et al.* (9) used plants which had been removed from a paddy field, and the exposures were performed in N-free nutrient solution. In this study, we used undisturbed plants grown and exposed in pots of soil.

Because experiments 1 and 2 showed no evidence of translocation of newly fixed N to the plant shoot, experiments 3 and 4 were designed to provide a more rigorous test for translocation. After the plant-soil system had been exposed to the $^{15}N_2$-enriched atmosphere, a single tiller was harvested and the rest of the plant was allowed to mature in a normal atmosphere before harvesting. The single tiller harvested immediately after the 13-day exposure period of experiment 3 was enriched with ^{15}N, and no enrichment was found in the tiller harvested immediately after the four day exposure in experiment 4 (Table III). Inasmuch as the shoot did not become enriched during any of the other exposures, the enrichment found at the end of the 13-day exposure is interpreted to be due to translocation of N fixed in the soil, roots, or lower shoot, and not due to phyllosphere fixation. The ^{15}N enrichment in the shoots increased with time, and at maturity, the shoots of the plants contained more of the total ^{15}N than the roots (Tables IV and V). In both experiments 3 and 4, the panicles which matured later were more enriched than the early maturing panicles. The pattern of increasing enrichment with time is compatible with the concept that the newly fixed N becomes available to the plant after it is incorporated into bacterial cells and subsequently mineralized.

Although the highest ^{15}N enrichments were found in the roots, the total quantity of ^{15}N incorporated into the subsurface soil was much greater in all four experiments (Tables I, II, IV, and V). This was most clearly shown in experiment 3 where the soil enrichments were sufficiently greater than the level of measurement error to allow reliable calculations (Table IV). This supports the earlier results of Yoshida and Ancajas (19) and Wada *et al.* (15) who found a similar pattern in the distribution of total acetylene reducing activity between roots and soil.

Our results show that a part of the nitrogen in a rice crop is derived from heterotrophic nitrogen fixation during the current crop, although the total amount is small. Using the data from experiment 3 (Table IV) and correcting for the atmospheric ^{15}N enrichment, N derived from fixation during the 13-day exposure was 0.49% of the total plant N. Incorporation of ^{15}N into the plant-soil system would be equivalent to 2.6 kg N ha^{-1} during the 13-day exposure, based on a ha-furrow-slice of 2×10^6 kg dry soil ha^{-1}. In a total N balance study using Maligaya soil treated in an analogous manner, App *et al.* (1) found that heterotrophic nitrogen fixation over six crops of rice was equivalent to 14% of the N removed in the grain and straw. Thus, it seems likely that the present study underestimates the potential contribution of heterotrophic nitrogen fixation.

Table V. *Experiment 4. ^{15}N Enrichment in a Rice-Soil System Exposed to $^{15}N_2$ for 4.1 Days (12:16 p.m. 63 DAT-2:00 p.m. 67 DAT, Heading Stage), and Harvested at Maturity (87 DAT).*

Oxygen concentration rose from 18–22% by day 2 and was lowered to 20% with the H_2 burner. By the end of the exposure, it had risen to 23%. Total N_2 remained at 25% throughout the exposure, but atom % ^{15}N decreased from 49–45% and Ar decreased from 56–52%.

Sample	Dry Wt	Total N	^{15}N	^{15}N	Total ^{15}N
	g	*mg*	*atom % excess*[a]	*mg*	*%*
Above black cloth					
Early panicles[b]	8.80	62.2	0.004 ± 0.001 (3)	0.003	0.6
Panicles	14.80	109.5	0.012 ± 0.001 (3)	0.014	2.6
Shoot	15.18	44.0	0.004 ± 0.003 (3)	0.002	0.4
Below black cloth					
Lower shoot	3.93	7.6	0.039 ± 0.002 (3)	0.003	0.6
Basal node	0.85	3.2	0.066 ± 0.002 (3)	0.002	0.4
Surface roots	0.28	1.7	0.119 ± 0.004 (2)	0.002	0.4
Subsurface roots	3.76	14.2	0.109 ± 0.007 (3)	0.016	3.0
Surface soil	169	106.6	0.068 ± 0.002 (4)	0.07	13.5
Subsurface soil	5740	3553.1	0.012 ± 0.002 (4)	0.43	78.7

[a] Values are relative to unexposed plant and soil samples. Standard deviations calculated with the number of samples indicated in parentheses.

[b] The early maturing panicles were harvested at 79 DAT.

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